Master's Thesis

Prey Phenotypic Changes During Long-Term Exposure to *Bdellovibrio bacteriovorus* HD100

SooIn Choi

Department of Biological Sciences

Graduate School of UNIST

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SooIn Choi

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Approved by

Advisor
Robert J. Mitchell

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This certifies that the thesis/dissertation of SooIn Choi is approved.

04/07/2017

Signature

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Advisor: Robert J. Mitchell

Signature

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Prof. Dougu Nam

Signature

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Prof. Cheol-Min Ghim

Prey Phenotypic Changes During Long-Term Exposure to *Bdellovibrio bacteriovorus* HD100

Abstract

Bdellovibrio bacteriovorus is gram-negative predatory bacteria who prey on other gram-negative bacteria. Once *B. bacteriovorus* meet their prey bacteria, they penetrate to periplasmic space and digest and absorb cytosolic contents of prey such as proteins, nucleic acids, and lipids. Because of their unique life cycle, many researchers believe these predatory bacteria could be next generation antibiotics. As they digest almost all cell structure when they invade in prey cell, researchers expected to *B. bacteriovorus* might not causing resistance on their predation. Previously, few researchers tried resistance development in prey cells against to predation, but they reported it is plastic. Thus, in this study, I wanted to try following prey survival and mutation to get predation resistance by exposing prey cells to *B. bacteriovorus*.

Usually, experiments related with *B. bacteriovorus* were performed no more than 72 hours, which means prey was exposed to predation situation only in 72 hours. To expose prey cells to *B. bacteriovorus* predation situation in order that I tried a batch and continuous culture. In both methods, prey cells decreased more than 4-log right after exposed to the predators, however, the number of prey cell recovered about 1-log and kept recovered population while predator cells present in media constantly. Interestingly, in continuous culture, the morphology of prey cell on the solid media had changed when they exposed to predator more than 5 days (120 hours). This morphological change of prey cell colonies was kept constantly even after storage in -80℃ or cultured in rich media.

From the microscopic image of phase changed colony from continuous culture, they usually showed smaller size compared with wild-type, about 60% of wild-type cell size. Moreover, in RNA-seq, about 250 genes related with the membrane, and flagellar were differentially expressed in phase changed colony compared with wild-type.

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Chapter 1. Introduction

1.1 Summary

Bdellovibrio bacteriovorus which is well known as a predatory bacteria prey on other gram-negative bacteria [1]. It exists naturally in nature, soil, water, and even inside the mammalian intestine [2-4]. *Bdellovibrio* and like organisms (BALOs) are the predator for other bacteria, invade into periplasmic space of other prey gram-negative bacteria, digest cytosolic contents, elongate, divide, and pop out from the envelope of prey cell [5, 6]. Because of this unique life cycle, BALOs are on the rise as a next generation antibiotics [7-13]. The one of side effect of antibiotics is resistance development [14]. However, as BALOs digesting whole cytosolic contents including nucleic acids, researchers expected no resistance develop against to predation. As BALOs able to attack a broad range of gram-negative pathogens, application for antimicrobial treatment seems possible. Although huge advantages are existed using BALOs as a next generation antibiotics there are limitations to actualize in a real site [15, 16].

1.2 Predatory bacteria: *Bdellovibrio* and like organisms

In nature, predator-prey relationship is commonly found a relationship between two different organisms. We could easily recall bear, lion, and alligator as a predator, gazelle, zebra, and rabbit as prey from the savanna. Likewise, the predator-prey relationship also exists in micro-world. One of well-known predator in microorganism world is *Bdellovibrio* and like organisms (BALOs). *Bdellovibrio* is gram-negative predatory bacteria prey on other gram-negative bacteria [1]. They are found from the broad common environment such as soil, water, and even in the mammalian intestine [2-4, 17]. BALOs usually demand other gram-negative bacteria for reproducing themselves. Once BALO cell invades into prey cell, it settles at periplasmic space where is between the outer membrane and inner cytoplasmic membrane making bdelloplast. Settled predatory bacterial cell at intraperiplasmic space digests cellular contents by secreting lots of lytic enzymes toward cytosolic space of prey cell. From digested nutrients, it elongates and divides to multiple daughter cells. Then, 3~6 daughter cells lyse outer membrane of prey cell and swim for finding new prey cell [5, 18].

1.3 Candidate as alternative antibiotics

From unique life cycle of BALO, many researchers propose BALO as living antibiotics to overcome antibiotic resistance [8-12, 14]. BALOs have many advantages to being used as some antibiotics. First, they could predate on many mammalian gram-negative bacteria [1, 11, 19]. For example, *A. baumannii* is one of the concerned human pathogens well known for the ability to develop multidrug resistance, but BALO could predate on *A. baumannii* whether it has resistance to antibiotics. Second, in that senses, even BALO predated multidrug resistance prey, as BALO digests whole cell contents of prey, shut off the possibility of multidrug resistance spreading through the horizontal gene transfer [5]. Third, even BALO cannot predate on gram-positive bacteria, lytic enzymes secreted by BALO could disperse biofilm formed by gram-positive bacteria, which makes difficult to treat antibiotics to bacteria inside the biofilm [20-24]. Lastly, as BALOs are already existing inside mammalian intestine [25], there will be no side effect which could be caused when using antibiotic.

1.4 Limitation to apply BALO as therapeutic agents

Although BALO has many advantages as antibiotics, many limitations also exist. Because of their unique life cycle, it also acts as obstacles for studying BALOs. There are many unknown parts of BALO, molecular features, mechanism of recognition and invasion, or etc. Moreover, such parasitic life cycle makes difficult to modifying BALO genome [26-28]. Another concern of applying BALO as therapeutic agents is that predation ability of BALO is challenged by many environmental factors, such as pH, temperature, salt concentration, viscosity, and so on [16]. And even the prey species range of each BALO strains are different [1]. For applying BALO as a real therapeutic agent, many problems including mentioned above should be considered and solved.

1.5 Resistance against predation

One of the obstacles of BALO as an antibiotic is that BALO does not predate on whole prey. The fact that BALO always remains few population of prey cells is reported several times. In 1979, Dr. Varon reported phenotypic changed prey after they exposed to predation in long-term [29]. In addition, in 2004, Dr. Jurkevitch tried to gather residual cells left after predation, and those residual cells were susceptible to predation again [15].

Chapter 2. Material and Methods

2.1 Strains and growth conditions

Escherichia coli str. MG1655 with plasmid pUCDK which confers bioluminescent, *Acinetobacter baumannii* clinical isolate with multidrug resistance, *Klebsiella pneumoniae* (ATCC13883, type strain), and *Bdellovibrio bacteriovorus* HD100 were used in the whole study. All were stored at -80℃ as frozen glycerol stocks. Upon need, *E. coli*, *A. baumannii* and *K. pneumoniae* were streaked on Luria-Bertani (LB, BD-Difco™, USA) agar plate and incubated at 37℃ overnight. And for the liquid cultured bacteria, each strain was cultured in LB broth inoculated from a single colony of a streaked agar plate and incubated at 37℃ overnight about 16-18 hours with shaking.

For streaking *B. bacteriovorus* HD100, it needs double layer plate. Bottom layer consisted of 1/10 diluted nutrient broth (DNB, Neogen, USA) with 1.7% agar containing 2mM of $MgCl_2$ and 2mM of $CaCl_2$, and top layer consisted of DNB with 0.7% agar containing 2mM of $MgCl_2$ and 2mM of $CaCl_2$. For preparing prey cells in liquid solution, 15ml of *E. coli* MG1655 overnight cultured cell in the 50ml conical tube (BD Falcon®, USA) were centrifugated (2,000×g, 15 minutes) and pelleted cells were resuspended in HEPES buffer. Before top layer agar was solidified, 2ml of overnight cultured *E. coli* MG1655 resuspended in HEPES buffer mixed with 8ml of molten top agar and poured on the bottom layer to solidify. Once prey cell containing top agar solidified, *B. bacteriovorus* were streaked on the top layer by scratching the solidified top agar. After incubation the double-layered plate at 30℃ for more than 2 days, small pieces of cleared zone around streaked line because of predation took for liquid culture. 1ml of HEPES buffer (25mM, pH 7.2, 2mM of $MgCl_2$ and 2mM of $CaCl_2$ added) mixed with *B. bacteriovorus* containing top agar pieces and filtered with a 0.45μm syringe filter (Merck Millipore LTD., USA) to get rid of residual prey cell and agar. The filtered solution was added to *E. coli* MG1655 containing HEPES buffer.

For routine sub-culturing *B. bacteriovorus* HD100 culture, the cleared predated culture filtered with 0.45m syringe filter and mixed with 15ml of *E. coli* MG1655 resuspended HEPES buffer of which optical density adjusted OD_{600} 1.0 (contains usually ~ 6×10^8 prey cells ml^{-1}) in 1:50 v/v ratio (300μl) in the 50ml conical tube. 16-18 hours overnight culture at 30℃ with shaking gave ~ 2×10^9 *B. bacteriovorus* cells ml^{-1} based on top agar plating plaque counting.

In the case of experiments that using *A. baumannii* and *K. pneumoniae* as prey cells, *B. bacteriovorus* HD100 was cultured with each prey cells from streaking on the double layer plate to routine sub-culturing.

B. bacteriovorus HD100 always cultured overnight more than 3 times before using the test as a liquid culture, and the culture was refreshed from -80℃ stock every 1-2 weeks.[21]

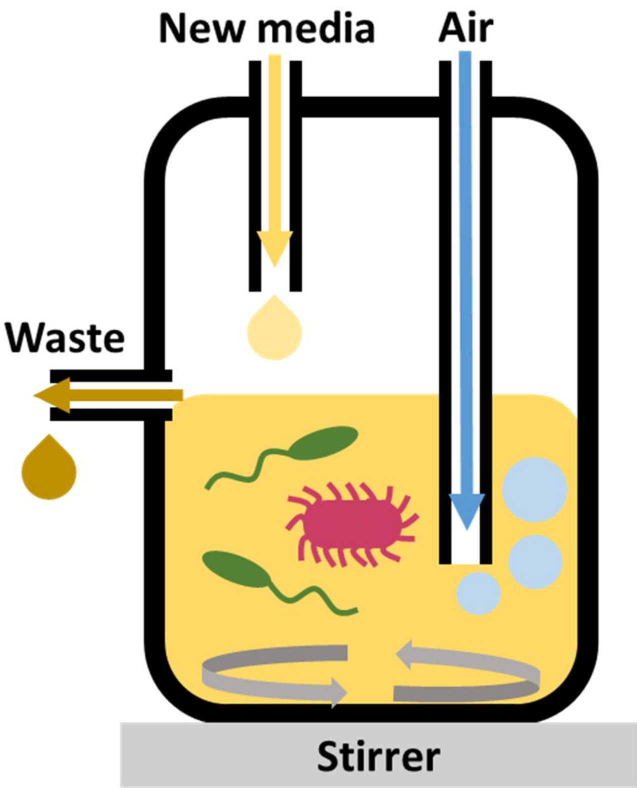
2.2 Long-term exposure to predation in batch culture

For getting a lot of prey cells, each prey cells were cultured 30ml in 100ml flask inside LB broth at 37℃. Overnight cultured prey cells were centrifuged (2,000×g, 15 minutes) and pelleted cells were resuspended in HEPES buffer adjusted to OD_{600} 1.0. 30ml of the suspension were distributed to two 100ml flasks. One of the flasks was for the control, without the predator, and the other was for predation. For the predation flask, overnight 16-18 hours cultured *B. bacteriovorus* HD100 with each prey was filtered with 0.45μm syringe filter. The filtrate was added to the predation testing flask in 1:50 v/v ratio (600μl of the filtered *B. bacteriovorus* HD100 culture). For checking population inside the prey-predator mixed solution in the flask, colony forming units (CFUs) and plaque forming units (PFUs) enumeration was used. CFU and PFU were checked every 24 hours.

2.3 Continuous culture system

From the previous research Dr. Varon at 1978, I mimicked her continuous system. (Figure 2.3.1.) [29] The temperature was kept at 30℃ by circulating 30℃ water through the space wrapped reactor by a water bath. The volume inside the reactor was approximately 100ml, and solution that over the 100ml was pumped out. Media was added using a pump to be diluted 0.1 $hour^{-1}$. That is, the contents of the reactor were refreshed within 10 hours. Air was supplied using air pump filtered with a 0.22μm filter (Merck Millipore LTD., USA) between the air pump and reactor. The magnetic bar was put inside the reactor in order that stirrer (500rpm, MS-MP4, DAIHAN-brand®, Korea) could mix the culture to make homogeneous. Once the system was established, I added 1ml of overnight cultured *E. coli* MG1655 with pUCDK and checked CFU and bioluminescence(BL) (GloMax® 20/20 Luminometer, Promega, USA) took from overflowed waste to confirm that prey settled inside the reactor stable. Antibiotics containing and not containing, two kinds of plates were used for check CFU, because the possibility that *E. coli* MG1655 pUCDK lost plasmid because of no antibiotics added media, and for monitoring contamination inside the reactor. CFU plates were incubated at 30℃ for kept temperature condition to prey cell constantly. If the CFU and BL kept similar level more than 24 hours, I added 1ml of filtered *B. bacteriovorus* HD100 overnight cultured about 16-18 hours. Dilution rate was checked by weighing the waste during a certain period, and sample for checking BL, CFU, and PFU was taken from the waste more than once a day.

(A)



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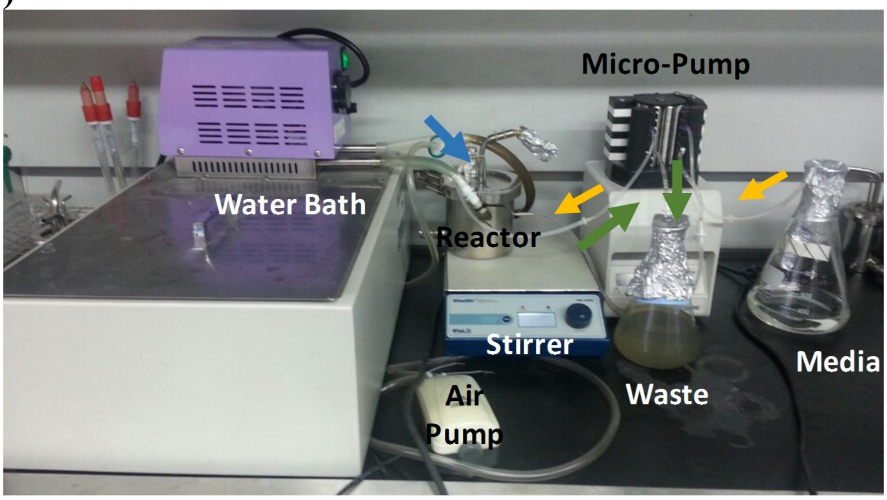


Figure 2.3.1. Scheme of continuous culture system. (A) Schematic view inside the reactor. (B) Overview of actual system. Media flowed into the reactor through yellow arrow direction while waste flowed out through green arrow. Air pumped into the reactor through blue arrow.

2.4 Testing reverting ratio of small colony variants

Small colony variants (SCVs) cells were kept in -80℃ frozen glycerol stock. To investigate the stability of SCVs phenotype, it was streaked on LB agar plate containing ampicillin (100μg/ml, Sigma-Aldrich, USA) and incubated overnight at 30℃. Single colonies from the plate were inoculated in 4ml of LB broth in 15ml conical tubes (BD Falcon®) and cultured 24 hours. After 24 hours, 40μl of the overnight culture was inoculated to 4ml of fresh LB broth in the 15ml conical tube. And the overnight culture was diluted and spread out on LB agar plate containing ampicillin (100μg/ml). CFU was counted differentially by their phenotype: wild type (translucent, flat) or SCVs type (opaque, raised).

2.5 Microscopic analysis of difference between wild type and small colony variants depends on growth phase

E. coli MG1655 as a wild type and SCVs were streaked on LB agar plate (ampicillin 100μg/ml) and incubated at 30℃ overnight. One single colony from each strain was inoculated into LB broth without any antibiotics and cultured overnight at 30℃. An overnight culture of wild type and SCVs were inoculated to fresh LB broth to adjust OD_{600} 0.03 and incubated at 30℃ with shaking. After about 4 hours, both cultures reached to OD_{600} ~ 1 and diluted to new LB broth adjusting OD_{600} about 0.03. Newly inoculated LB broth incubated at a 30℃ shaking incubator. Every 1 hour, 1ml of each culture was taken, centrifuged, and resuspended with 200μl 4% paraformaldehyde. After 8 hours when both strains entered into stationary phase, cells were stained by SYTO™ 9 Green Fluorescent Nucleic Acid Stain (absorption 485nm, emission 498nm, Invitrogen™, USA). Stained cells were taken images using confocal laser scanning microscope (IX81, Olympus, USA) operated with ZEN (MetaMorph®, Germany).

2.6 RNA preparation for RNA-seq

E. coli MG1655 pUCDK as a wild type and SCVs were streaked on LB agar plate (ampicillin 100μg/ml) and incubated at 30℃ overnight. Three different single colonies for each strain from the plate were inoculated into LB broth without any antibiotics and cultured overnight at 30℃. The overnight culture of wild type and SCVs were inoculated to fresh LB broth to adjust OD_{600} 0.03 and incubated at 30℃ with shaking. After about 4 hours, both cultures reached to OD_{600} ~ 1 and diluted to new LB broth adjusting OD_{600} about 0.03. Newly inoculated LB broth incubated at the 30℃

shaking incubator and checked OD_{600} every 1 hour. After 2 hours from the inoculation, both cultures reached about OD_{600} ~0.3. 2ml of each culture centrifugated (16,000×g, 1 minute). Pelleted cells were used for RNA extraction using ChargeSwitch® (Invitrogen, USA). The RNA concentration was measured using BioPhotometer plus and μCuvette® (Eppendorf, Germany). The RNA quality was assessed using agarose gel (PhileKorea Technology INC., molecular biology grade) with RNA Gel Loading Dye (Thermo Scientific™, USA). For prevent RNA degradation, RNaseOUT™ (Invitrogen™, USA) was used.

2.7 RNA-seq analysis

Before sequencing, RNA was assessed by 2100 Bioanalyzer (Agilent Technologies, USA). RNA sequencing conducted on Illumina HiSeq 2500 platform. The whole procedure was performed by ChunLab Inc. (Seoul, Korea). RNA-seq total read count data was assumed as negative binomial distribution. It was normalized by Trimmed Mean of M-values (TMM) method [30] and calculated fold change by logarithm base 2. Using edgeR, differential expression analysis was performed and the significance (q-value) for each gene was evaluated by False Discovery Rate (FDR), Benjamini-Hochberg (BH) procedure [31]. Information related to each gene was found at *EcoCyc* (http://EcoCyc.org), a biological database for the *E. coli* K-12 MG1655 strain [32].

Chapter 3. Experimental Results

3.1 Long-term exposure to predator in batch culture

Before starting to perform predation for long-term, the influence of media enrichment to predation and prey survival was evaluated. From HEPES buffer as poor media to NB as rich media were used as media. Same *E. coli* MG1655 overnight culture was centrifugated and resuspended with different media. As adjusted OD600 1.0 prey population was ~6×10^{8} ml^{-1} while overnight cultured *B. bacteriovorus* HD100 population was ~1.8×10^{9} ml^{-1}. Then predator cells added to prey cell resuspension by each media in 1:50 v/v ratio which gave a predator-prey ratio (PPR) of 0.07. The changes in media were confirmed with CFU enumeration. (Figure 3.2.1.) The predator-prey mixed culture incubated for 72 hours and prey cells in each media decreased until 24 hours and recovered their population more than 1-log. In the case of HEPES buffer, which was poor hypotonic media showed most clear predation and reestablishing of prey cell population about 2-log increasing. On the other hand, in the case of media containing nutrient, it seemed prey cells were survived more from predation, less than 4-log decreasing prey cells with DNB, 0.5X NB, and NB while 6-log decreasing with HEPES buffer. Furthermore, population recovery in nutrient containing media was less than HEPES buffer - around 1-log increase. Therefore, HEPES buffer was used for the further study to observe predation and prey cell recovery clearly.

Based on above test, prey cell reestablishment after predation was tested with three different strains, *E. coli* MG1655, *A. baumannii*, and *K. pneumoniae* in HEPES buffer. *A. baumannii* and *K. pneumoniae* were selected because they are well known as multidrug resistance developed strains [33, 34]. Changes inside the prey-predator mixed culture were followed by measuring optical density (600nm) and counting CFU/PFU. (Figure 3.2.2.) As shown in the Figure 3.2.2., all three strains showed population reestablishment after more than 48 hours predation showing more than 2-log increase from the lowest cell number, under the existence of a high concentration of predator cells in same media. One remarkable thing was that OD600 value did not correlate with actual population inside culture solution once predation was done that OD600 drops the lowest value.

In the case of *A. baumannii* and *K. pneumoniae*, different from *E. coli*, they developed phenotypic changes when they exposed to predation more than 48 hours. (Figure 3.2.3.) Both strains usually show opaque, raised morphology of colony on LB agar plate. However, they changed their morphology to translucent, flat colony after more than 48 hours from the predator addition, which did not show at the control group plate, which incubated *A. baumannii* and *K. pneumoniae* without predator in HEPES buffer.

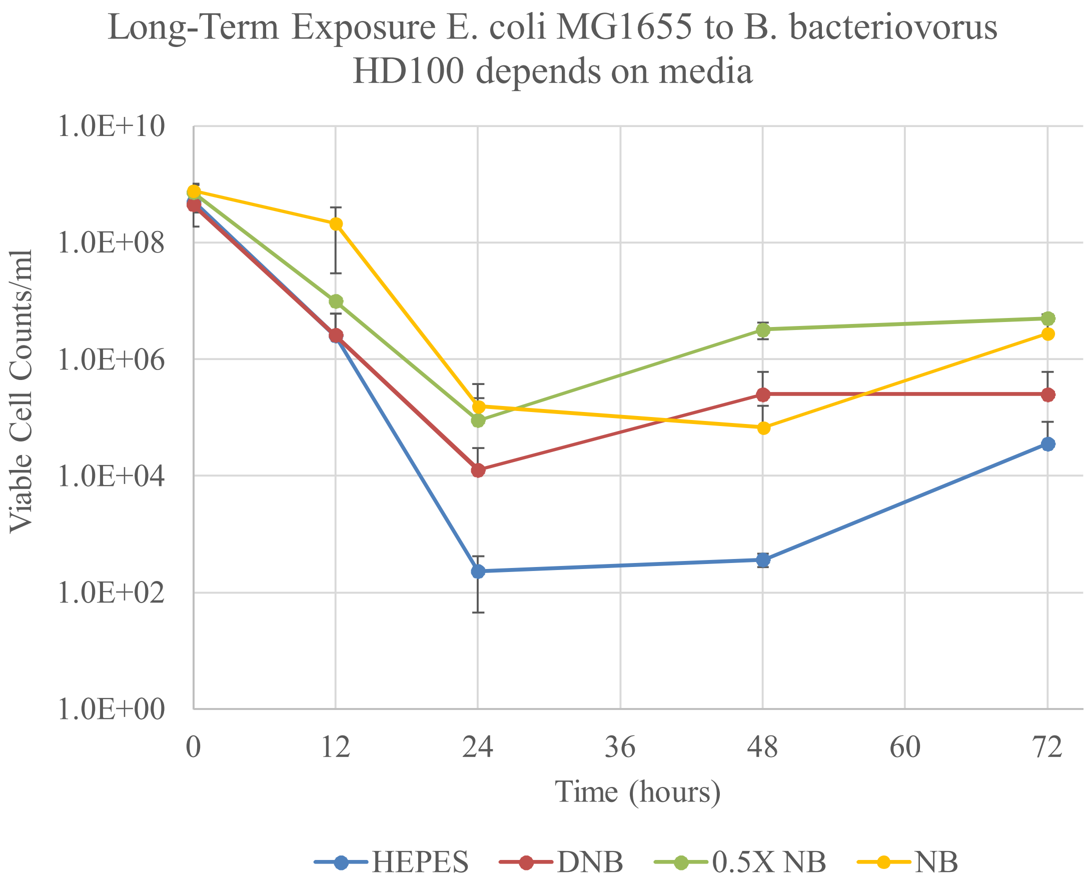
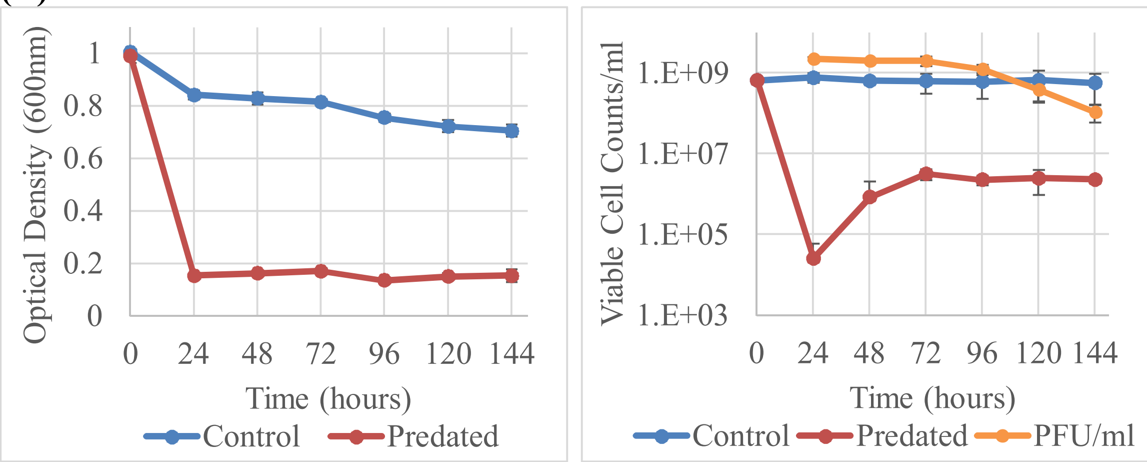


Figure 3.2.1. Long-term exposure *E. coli* MG1655 to *B. bacteriovorus* HD100 predation depends on media. Prey cells were mostly predated when prey cells were resuspended in HEPES buffer. (PPR: HEPES=0.039, DNB=0.044, 0.5X NB=0.027, NB=0.026)

(A)



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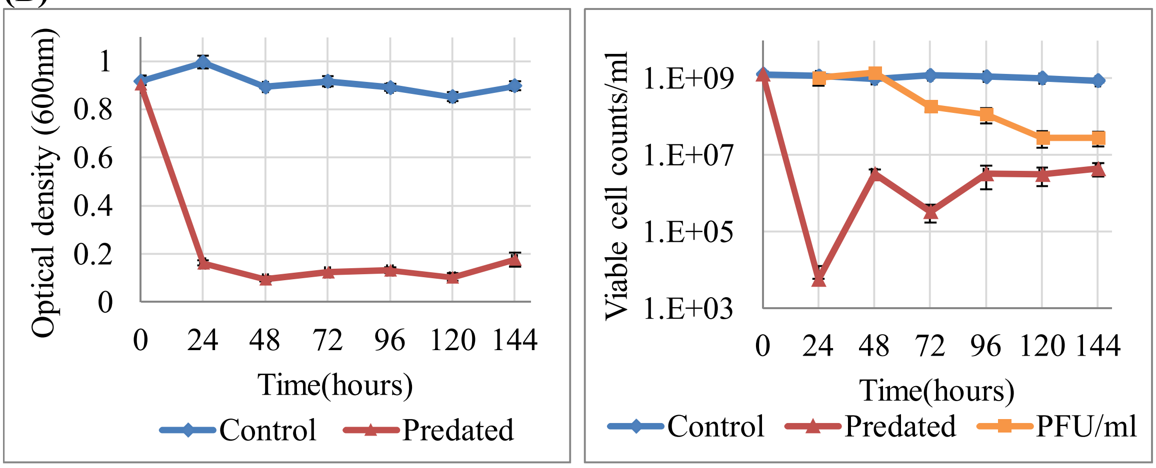

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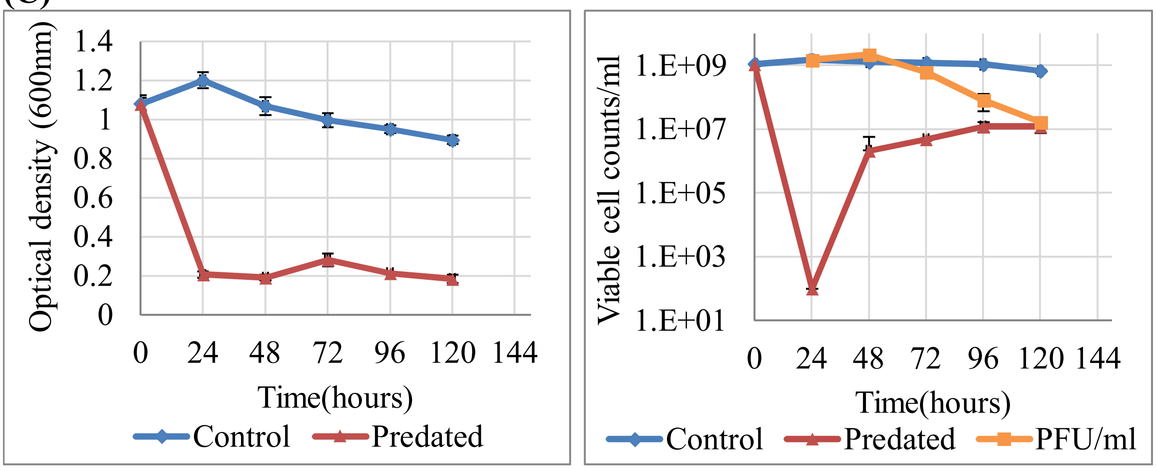
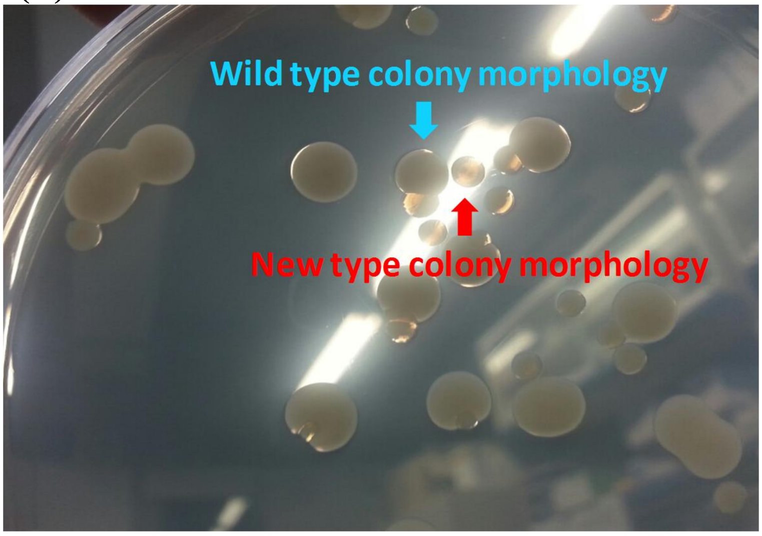


Figure 3.2.2. Long-term exposure prey to *B. bacteriovorus* HD100 predation in batch culture. (A) *E. coli* MG1655 (PPR=0.07) (B) *A. baumannii* (PPR=0.004) (c) *K. pneumoniae* (PPR=0.005)

(A)



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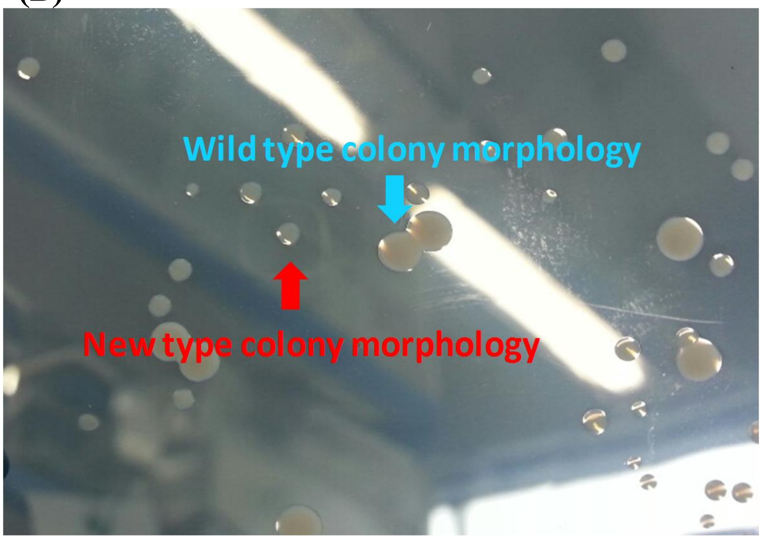


Figure 3.2.3. New type colony morphology of prey after long-term exposure to predation. (A) *A. baumannii* development of new type colony morphology. Wild type colony morphology is opaque, raised, and steaky, while new type colony morphology is translucent, flat, and grow slower. (B) *K. pneumoniae* development of new type colony morphology. Wild type colony morphology is opaque, raised, and steaky, while new type colony morphology is translucent, flat, and grow slower.

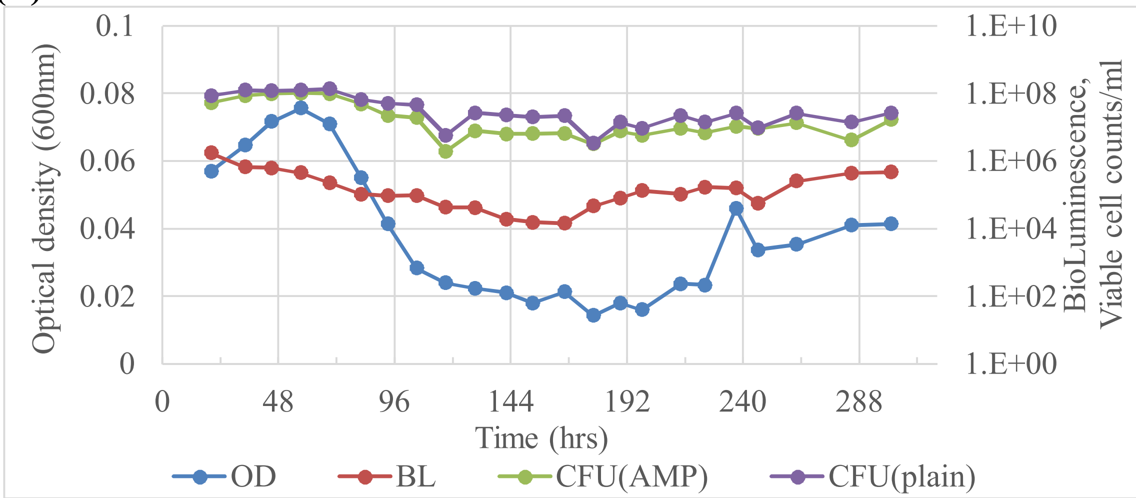
3.2 Long-term exposure to predator in continuous culture

Batch culture is a closed system so long-term cultivation with limited nutrients and accumulation of metabolic wastes could affect on a long-term experiment to observe predator-prey relationship. So, there is another way to cultivate bacterial cells, continuous system, which is adding media and wasting overflow for making chemically equilibrium status. Such characteristics of the continuous system, I chose a continuous system for long-term predation exposure experiment. As *E. coli* MG1655 did not develop phenotypic changed colony morphology, I selected *E. coli* MG1655 as a prey for a continuous system.

First of all, as optical density does not correlate with prey cell population, *E. coli* MG1655 with pUCDK which encodes luciferase gene expressing bioluminescent (BL). Thus, *E. coli* MG1655 pUCDK tested for sure of plasmid stability culturing long-term without antibiotics. (Figure 3.3.1.) Three independent reactors were run just with *E. coli* MG1655 with plasmid pUCDK. Two kinds of CFU plate was used, plasmid stability was evaluated by comparing the number of colonies appeared on antibiotics containing and not containing plates. 96 hours from system operation stated, the number of colonies between antibiotics containing and not containing plates showed some difference, but no more than twice the difference. Moreover, during more than 200 hours run (Figure 3.3.1. A), the difference between the number of colonies on antibiotic containing and not containing plates was still less than twice. In addition, prey cells kept their population level around 1×10^8 ml^{-1}, and BL also kept constant level around 1×10^5, while optical densities were different every time in range 0.02 to 0.08. Therefore, I decided to use BL value to estimate cell population inside the reactor.

Then I run the continuous culture with prey and predator. 16-18 hours overnight cultured *E. coli* MG1655 pUCDK added first, and CFU checked more than 24 hours to confirm the prey cells were established stable and there was no contamination by other bacteria. For the next, filtered by 0.45μm syringe filter *B. bacteriovorus* HD100 16-18 hours overnight culture was added to the reactor that containing prey cells. (Figure 3.3.2) Different from previous research by Dr. Varon, predator and prey inside the reactor kept their population constantly more than a week, not oscillating, similar with the batch culture results. Prey population was kept around 1×10^5 ml^{-1}, with BL value also around 1×10^5, while predator cells were kept their population at 1×10^8 ml^{-1}. Nevertheless, prey exposed to predation about 5 days showed phenotypic changes, translucent, flat colony morphology was changed to opaque, raised shape. (Figure 3.4.1.)

(A)



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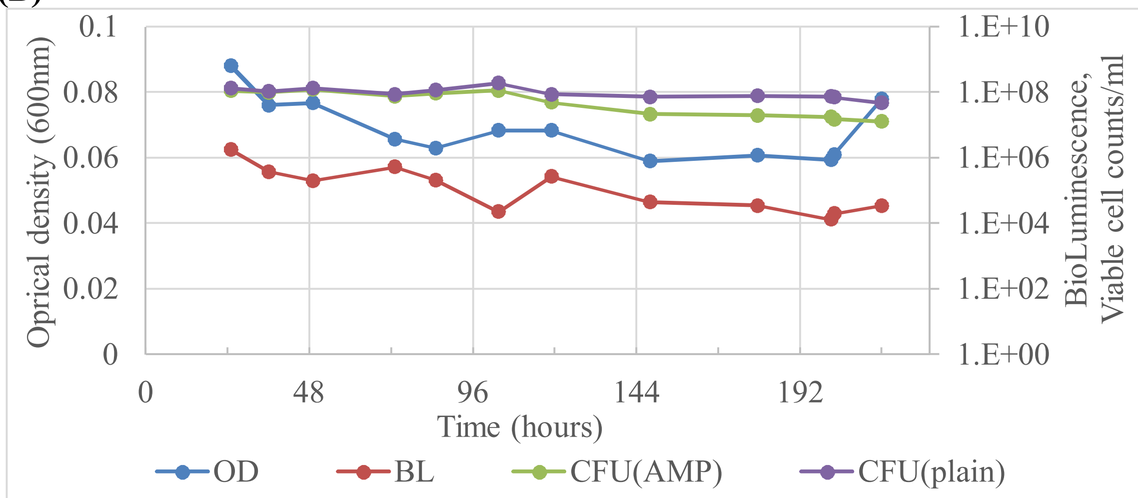

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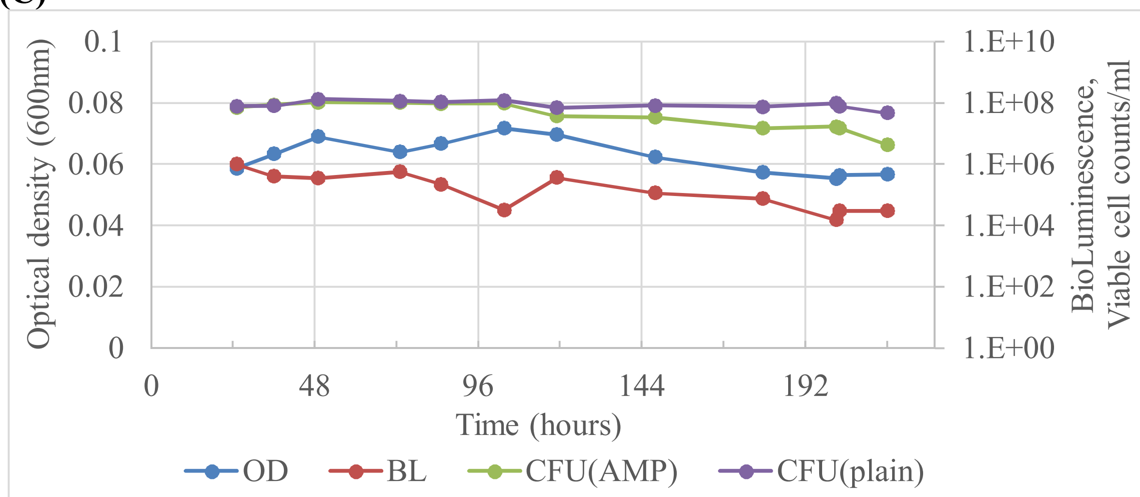
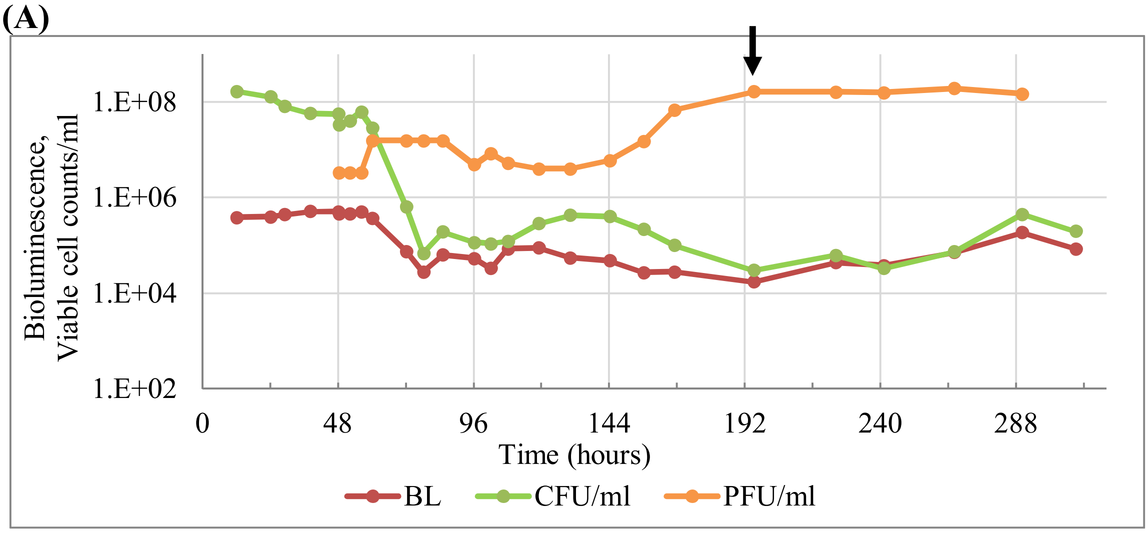


Figure 3.3.1. Plasmid pUCDK stability inside continuous system without antibiotics

(A)



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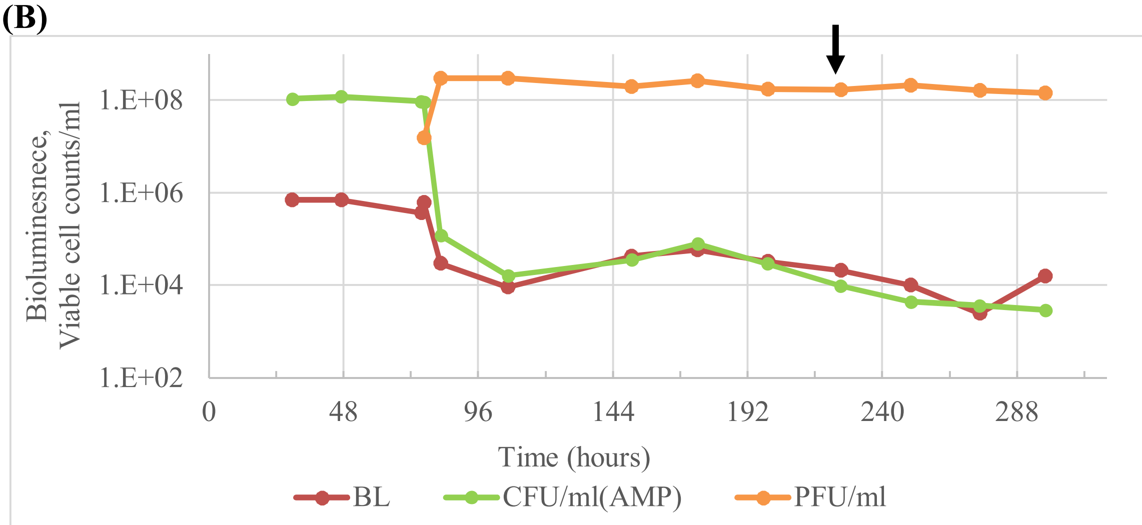

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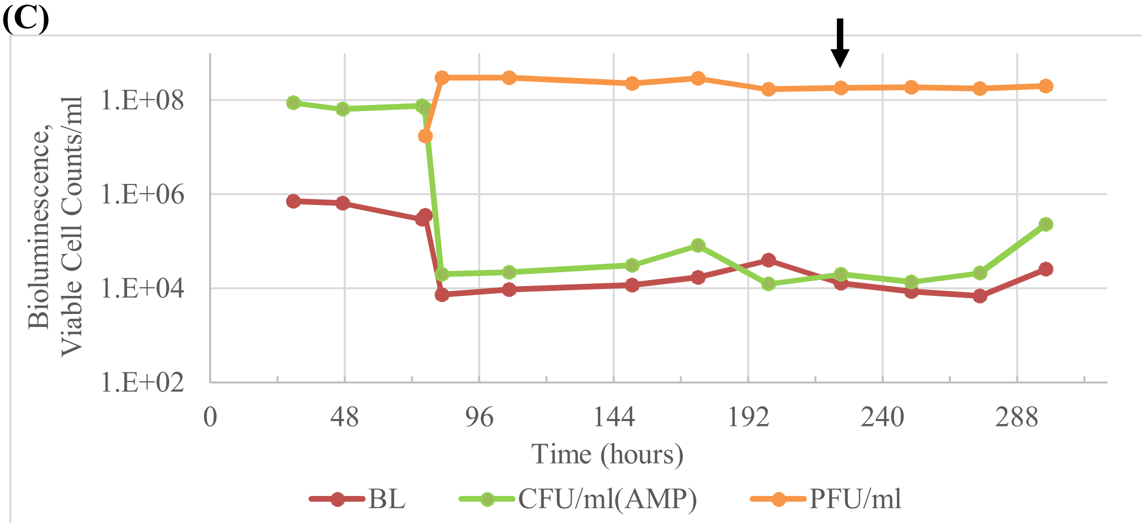


Figure 3.3.2. Long-term exposure *E. coli* MG1655 pUCDK to *B. bacteriovorus* HD100 predation in continuous culture system.

3.3 Characteristics of Small Colony Variants (SCVs)

The newly appeared type of colonies after exposed to predation more than 5 days was commonly showed smaller size than wild-type when they incubated in the same time, I named the new type colonies as “Small Colony Variants (SCVs)”. (Figure 3.4.1.)

For sure about the SCVs had stably changed phenotype, reverting ratio was tested. (Figure 3.4.2.) Overnight cultured SCVs were plated for counting SCVs and revertant population ratio every 24 hours. 1st or 2nd days, there was no reverted colony. After a 3rd day from the culture, few colonies showed wild-type morphology, translucent and flat. The number of reverted colonies were not over the 30% of total colonies, so I concluded the phenotypic change of SCVs is stable.

One of the important features between two different bacterial species is growth curve and doubling time. As tested above, SCVs were confirmed about their phenotypic stability, so growth curve was depicted depends on optical density (600nm). Under the same condition, 30℃, LB broth, with shaking, wild-type *E. coli* MG1655 pUCDK and SCVs were cultured and measured optical density (600nm) every 1 hour. (Figure 3.4.3.) As shown in the growth curve (Figure 3.4.3. A), SCVs were grown slower from early exponential phase, started after 3 hours from inoculation, and not grown more than OD_{600} around 1.5 while wild-type entered stationary phase around OD_{600} 2.5. From the growth curve, doubling time was calculated during exponential phase, 2 hours to 5 hours from inoculation. Wild-type needed 47.68 minutes to double their cell, whereas SCVs needed 57.63 minutes for doubling. That is, SCVs needed 10 minutes for double their size and population.

For comparing their phenotypic changes at single cell scale, they were observed under the confocal microscope. While wild-type and SCVs were cultured for measuring growth rate, 1ml of cells were taken for images in every 1 hour. They were fixed immediately after taken from the culture, and stained with fluorescent dye. After all, samples were gathered from 1 hour to 8 hours incubated for each cell, wild type and SCVs, images were taken with a confocal microscope. (Figure 3.4.4.) At the initial stages, 2 and 3 hours, both cells showed rod shape, and most of the cells were elongated or dividing. The average sizes of each cell were 6.05μm of wild-type and 3.96μm of SCVs, which showing 2/3 smaller size compared to wild-type. When the time passed to 4-6 hours, SCVs cells started to change their shape round, although wild-type still showed rod shape. After more than 7 hours passed, both wild type and SCVs showed round shape, but the average sizes were different about 2/3 small of SCVs (wild type = 3.67μm, SCVs = 2.76μm)

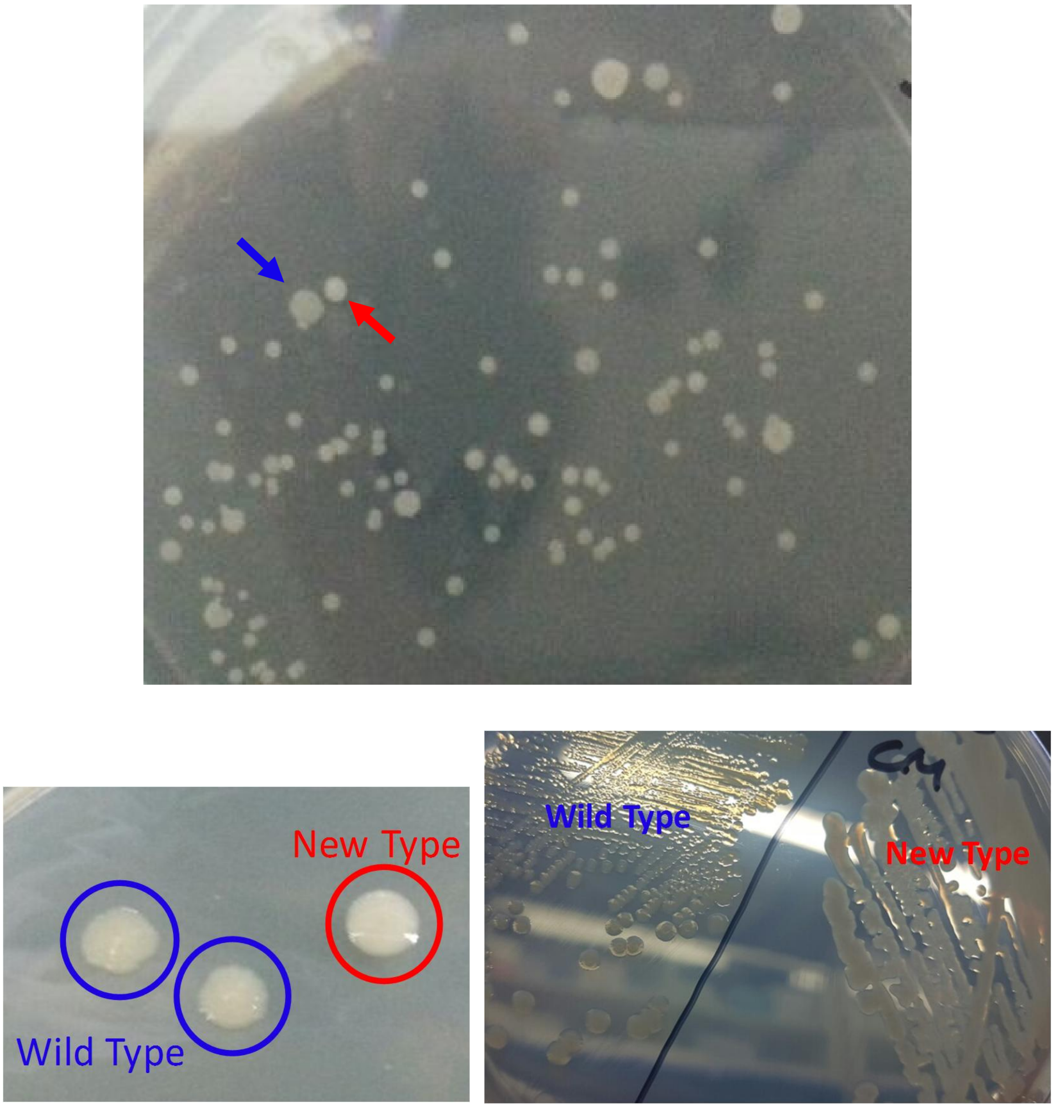


Figure 3.4.1. Small Colony Variants (SCVs) from long-term predation exposure in continuous culture system. Wild type colonies usually show translucent and flat colonies, while SCVs are growing slower, and have opaque, raised and waxy phenotype as a colony. Blue arrow pointing wild type colony. Red arrow pointing new type colony, small colony variants (SCVs)

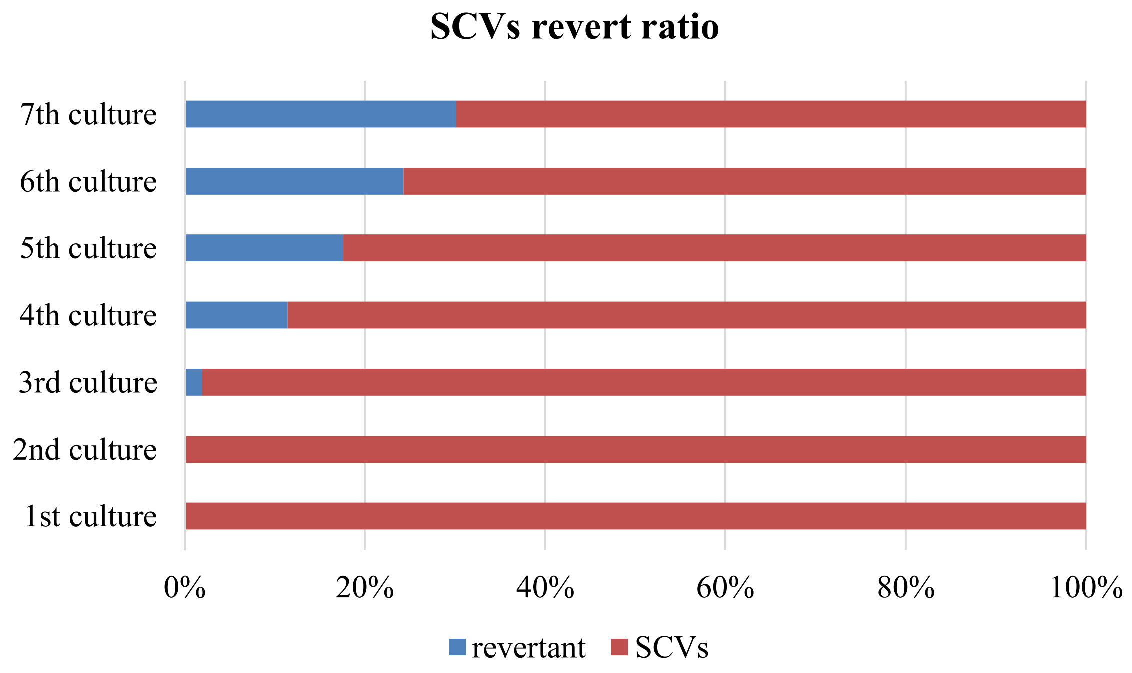
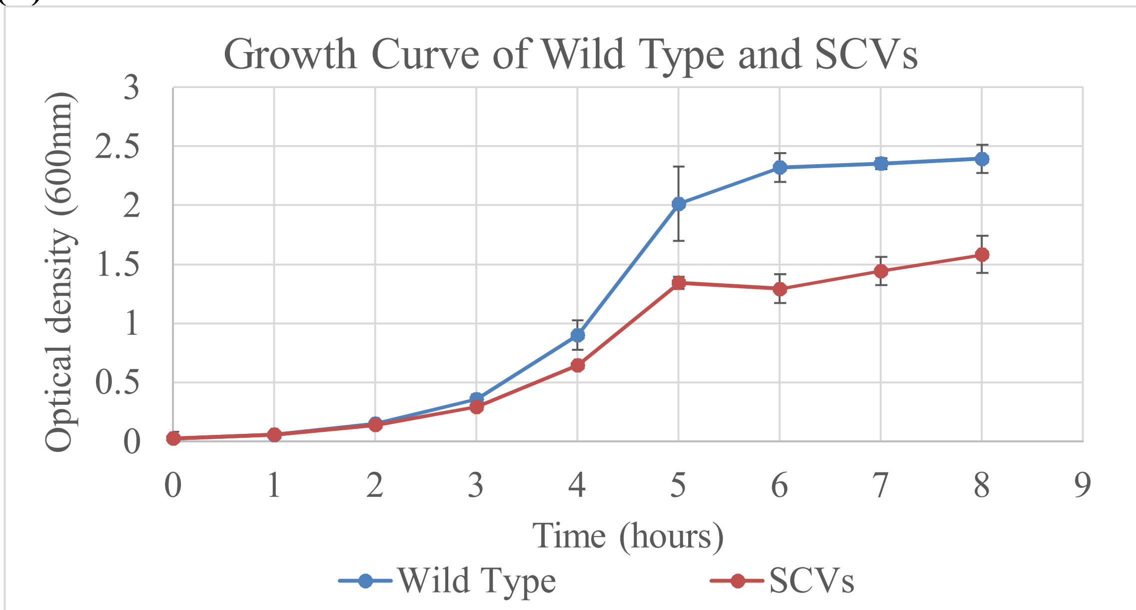


Figure 3.4.2. Revert ratio of SCVs to wild type phenotype. At the first, SCVs were streaked on LB agar plate with ampicillin for selection, incubated at 30℃ overnight. Three different SCVs colonies were picked and inoculated to LB broth 15ml in 50ml conical tubes without antibiotics, incubated at 30℃ overnight. That was a 1st culture, diluted and plated for counting revertant ratio. Overnight culture inoculated 150μl to new LB broth 15ml (1/100 dilution) and overnight cultured at 30℃. Every overnight culture was inoculated to new LB broth and plated on LB agar for counting revertant ratio.

(A)



(B)

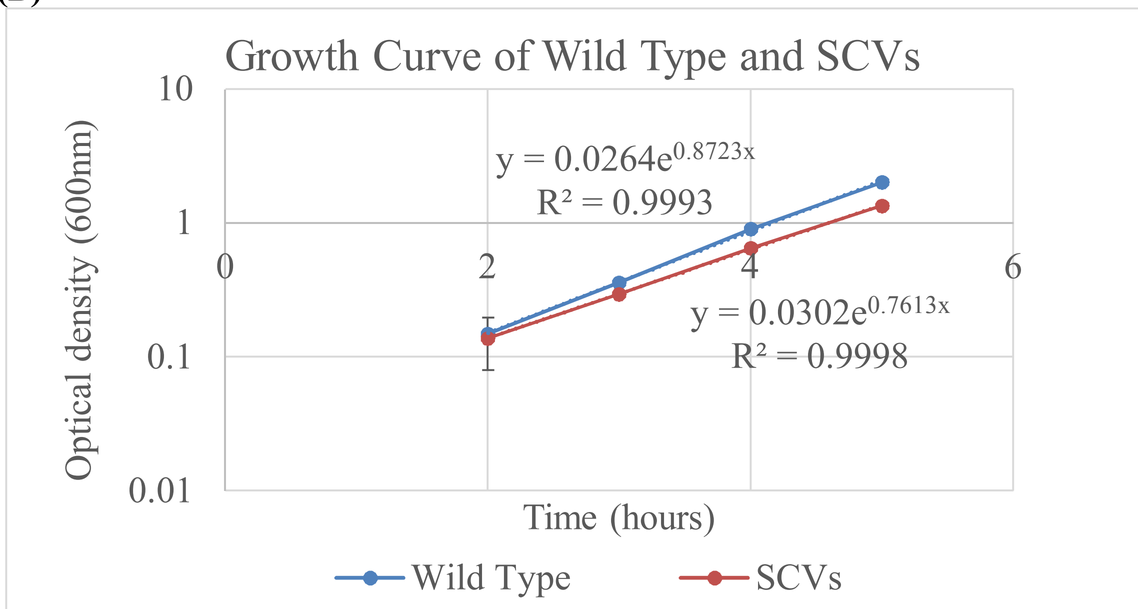


Figure 3.4.3. Growth curve of wild type and SCVs. They were incubated in LB broth at 30℃ shaking incubators. (A) Growth curve of wild type and SCVs. SCVs were not grow more than OD_{600} 1.5. SCVs were growing slower and entered to stationary phase at low optical density. (B) During exponential phase, wild types needed 47.68 minutes for double their population, on the other hands, SCVs took 57.63 minutes for double their population.

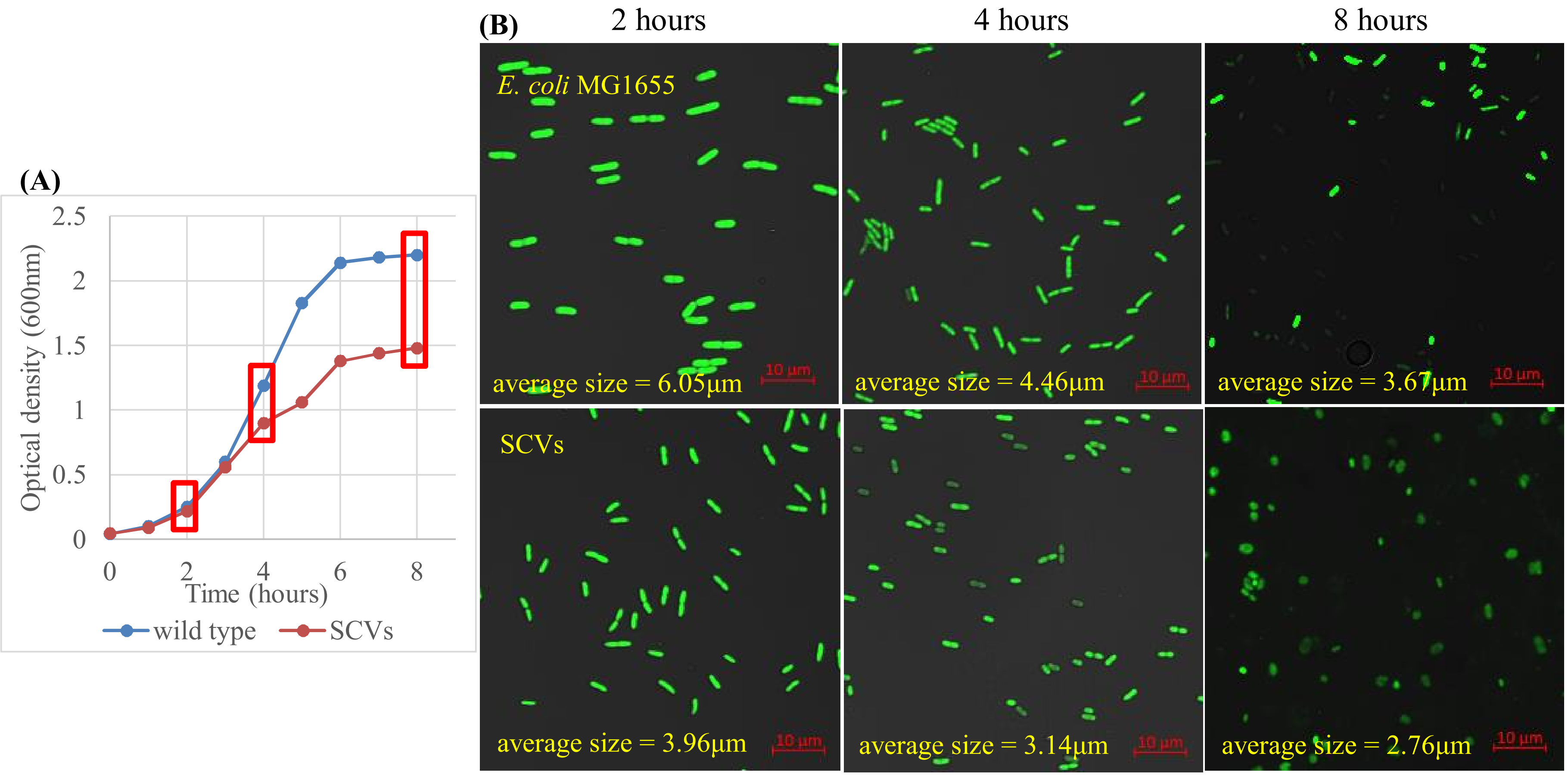


Figure 3.4.4. Confocal microscope image of wild type and SCVs depends on growth phase. Time points for taking image were selected by growth curve (A), depends on their growth stage. (A) Growth curve for each colony type, wild type and SCVs. Three time points were selected, 2 hours (OD600 both about 0.03, early exponential phase), 4 hours (OD600 0.8~1.2, middle of exponential phase), and 8 hours (stationary phase). (B) Confocal images took with LIVE® staining. Sizes of cells at each stage always smaller in case of SCVs. (n=15)

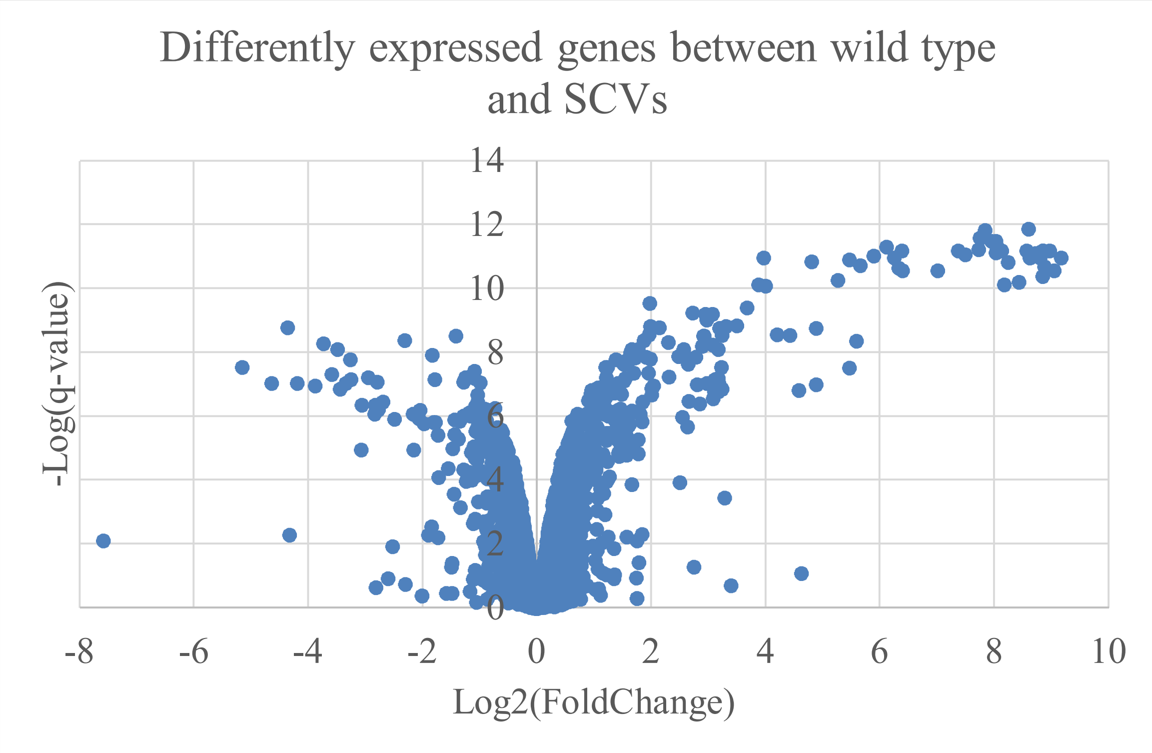
3.4 Transcriptomic analysis about SCVs

From the microscopic image, at the early exponential phase, they just showed size difference under the microscope. Also, at the growth curve, they showed different growth rate after that point. So, I chose the early exponential phase for analyzing transcriptomic difference which making further phenotypic changes between wild-type and SCVs. RNA was extracted proper growth condition with three independent culture for each cell type.

RNA was assessed and sequenced by ChunLab. The sequenced result was normalized by TMM. Normalized genes were compared by q-value and fold change. The differently expressed genes were plotted with q-value and Log_2(FoldChange). (Figure 3.5.1.) Fold change was compared to an average of read counts of each gene (SCVs/wild type). After that, certain genes were selected by more than 2-fold change in both sides, and a q-value less than 0.05. (Figure 3.5.1. B)

Among the genes selected above, I categorized the genes based on their function. (Figure 3.5.2) Overexpressed genes in SCVs compared with wild type could be categorized fimbriae, *rpoS*, and energy generation. In the case of *fim* group, genes were reported causing phenotypic changed in *K. pneumoniae*, which is known as related to their encapsulation [35]. Moreover, in the case of *rpoS*, which is known as a stationary phase and stress responses related sigma factor. *yjb* and *wca* are related with extracellular polysaccharides synthesis, and *osm* related to osmotic stress, known for changing outer membrane lipoprotein that may cause cell aggregation which is in the regulon of *rpoS*. In addition, *kdp* and *ent* are related to energy generation. On the other hand, *flg* and *fli* are less than two-fold decreased expressed which are related to flagellar, and *waa* is also expression decreased gene group related to lipopolysaccharide synthesis. Particularly, *omp*F and *omp*C were both downregulated which respond to stationary phase, osmotic stress, relate with *rpo*S overexpression, although the RNA was extracted at early exponential phase [36]. The most interesting result was about *flu* gene which was highly overexpressed in SCVs, known for the molecular switch of phase variant in *E. coli* [37]. From the above facts, I guess the membrane composition was changed in SCVs causing opaque, raised colony phenotype.

(A)



(B)

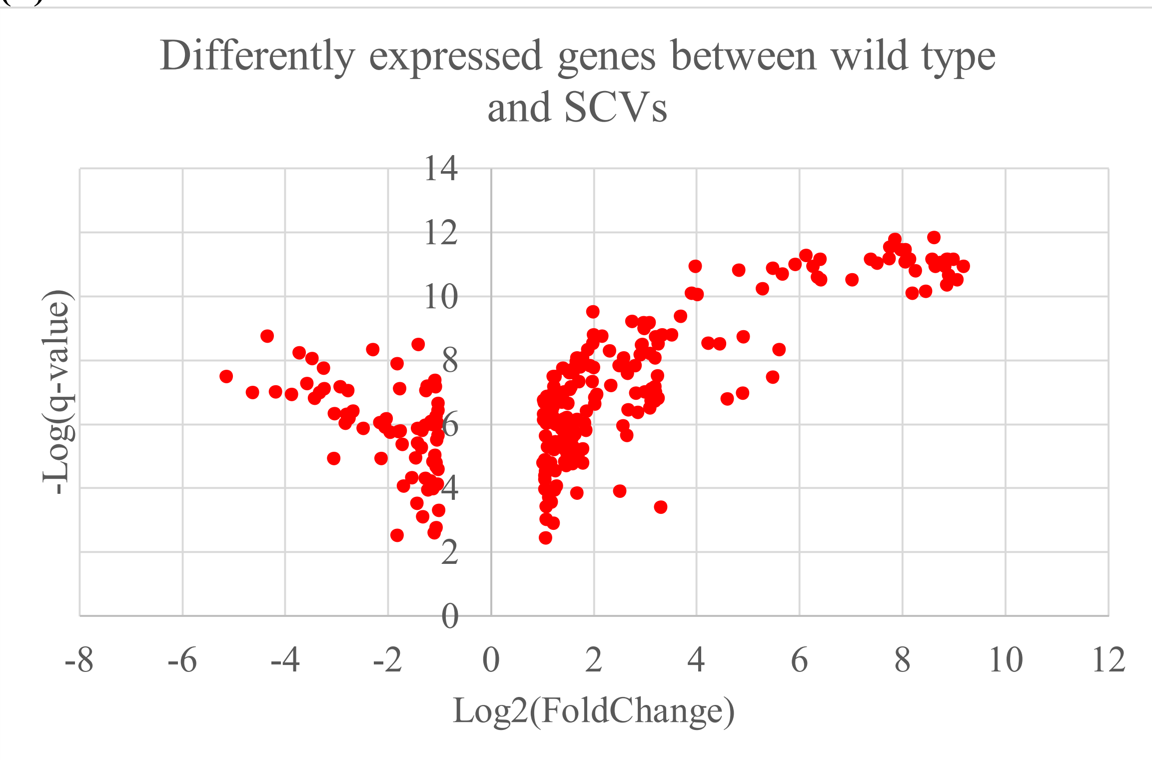
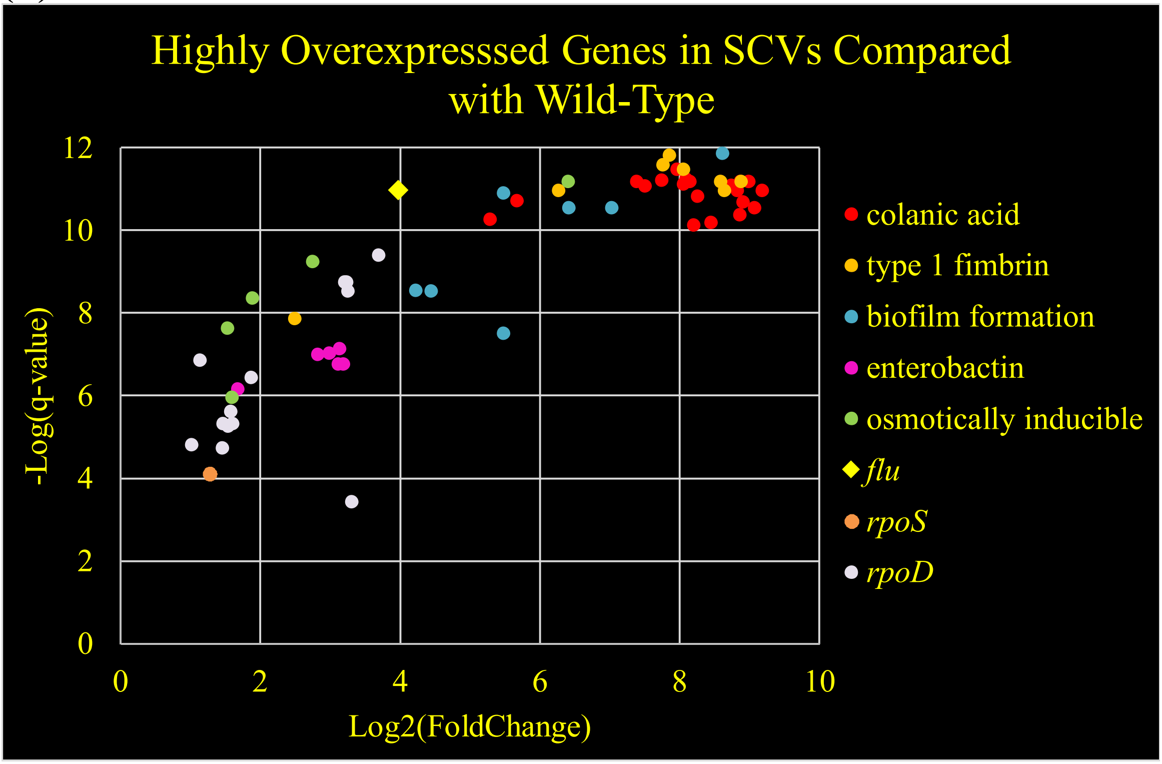


Figure 3.5.1. Differently expressed genes between wild type and SCVs based on RNA-seq. (A) x-axis=log2(SCVs RNA read count/wild type RNA read count). y-axis=-log(q-value). 4498 genes are plotted based on RNA read count assumed as Negative Binomial Distribution. (B) 252 genes were filtered by q-value < 0.05 and |log2(SCVs/wild type)| > 1.

(A)



(B)

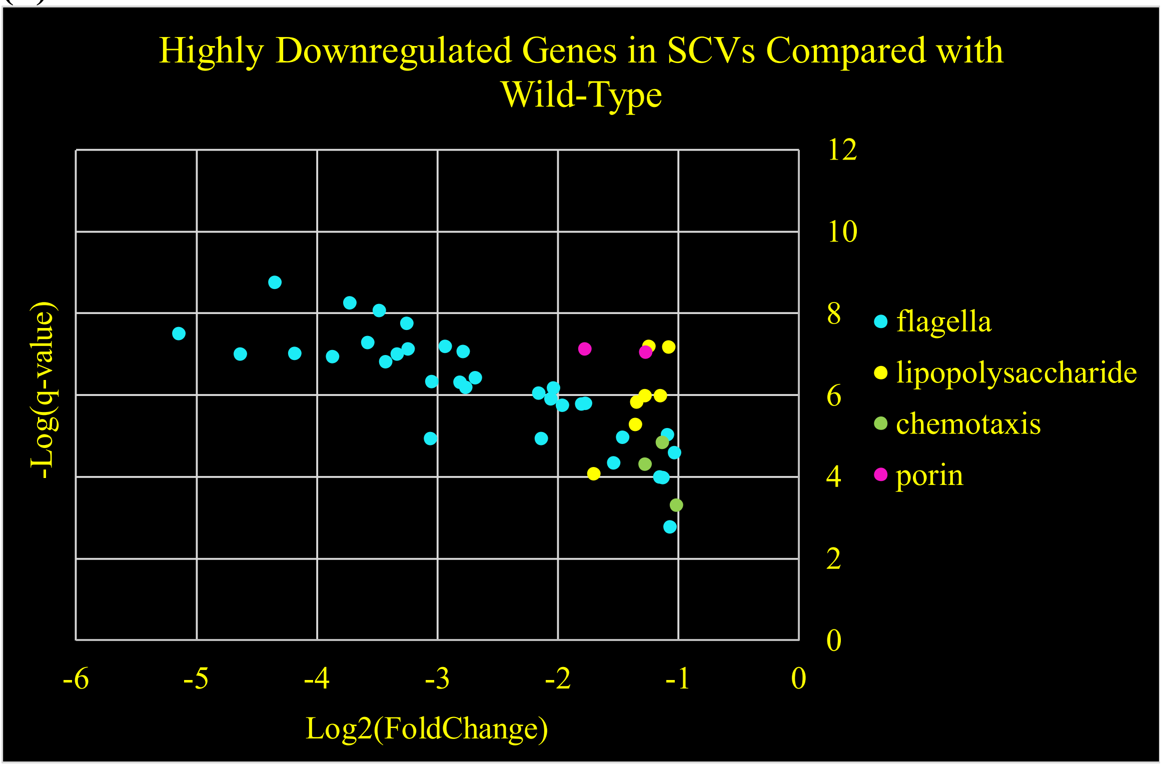


Figure 3.5.2. Significantly distinctly expressed gene between wild type and SCVs. (A) SCVs gene groups which were overexpressed compared with wild types. (B) SCVs gene groups which were less-expressed compared with wild types.

Chapter 4. Discussion & Conclusion

This study is focused on the prey response when they are exposed to predation in long term. When *E. coli* MG1655 exposed to predation by *B. bacteriovorus* HD100, although they predated more than 99.9% of their population within 24 hours, they reestablished their population more than the 2-fold increase from the lowest population. Likewise, *A. baumannii* and *K. pneumoniae* showed population recovery after significant population loss by predation of *B. bacteriovorus* HD100. Moreover, in the case of *A. baumannii* and *K. pneumoniae* showed phase variant after 48 hours from predation exposure. Such phenotypic changes were reported previously in both *A. baumannii* and *K. pneumoniae* strains. Previous researchers suggested that phase variants are caused by changes in surface structures, such as fimbria, flagella, and lipopolysaccharides [35, 38]. One remarkable thing is that in the case of *A. baumannii* and *K. pneumoniae*, *B. bacteriovorus* HD100 showed 2-log decrease started from 72 hours predation while *B. bacteriovorus* HD100 predated on *E. coli* MG1655 about 1-log decrease showed more than 120 hours from predation started. Our predatory bacteria *B. bacteriovorus* HD100 is known as immortal under the starving condition for 120 hours [39], so early population decrease of predator in *A. baumannii* and *K. pneumoniae* culture may have the factor that causing a phenotypic change of prey and death of predator.

Dr. Varon showed phenotypic changed prey cells in her continuous culture with prey (*photobacterium leiognathi* E28) and predator (*B. bacteriovorus* BM4) together. Similarly, in my continuous system, with *E. coli* MG1655 and *B. bacteriovorus* HD100, phenotypic change of prey colonies appeared. In the case of Varon's variants, they were becoming smaller, grow slower, less predated, and less luminescent. SCVs were showed similar phenotypic change, smaller and grow slower [29]. Mainly, it is because prey strain was different, and predator strain was different. However, becoming smaller and growing slower are sharing phenotypic changes which might suggest that predation pressure makes the prey cell metabolism inhibited.

Transcriptomic analysis revealed that many differentially expressed genes in SCVs are related with surface structure. According to such a difference between genes, for example, *wca* and *waa* are both related to extracellular polysaccharide synthesis but *wca* is overexpressed while *waa* is less expressed compared with wild-type, suggesting that the composition of the outer membrane is changed in SCVs which might relate to the morphological change to opaque.

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